Biochimica et Biophysica Acta 1852 (2015) 2414–2422

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

CD36 initiated signaling mediates ceramide-induced TXNIP expression in pancreatic beta-cells

Udayakumar Karunakaran [a], Jun Sung Moon [b,*], Hyoung Woo Lee [b], Kyu Chang Won [a,b,**]

[a] Institute of Medical Science, Yeungnam University College of Medicine, Daegu, South Korea
[b] Department of Internal Medicine, Yeungnam University College of Medicine, Daegu, South Korea

ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form 13 August 2015
Accepted 17 August 2015
Available online 20 August 2015

Keywords:
Diabetes
Beta-cell dysfunction
Ceramide
CD36
TXNIP
NF-κB

ABSTRACT

Diverse mechanisms are involved in the pathogenesis of β-cell failure in type 2 diabetes. Of them, the accumulation of ceramide, a bioactive lipid metabolite, is suggested to play a major role in inflammatory and stress responses that induce diabetes. However, the downstream inflammatory target of ceramide has not been defined. Using rat islets and the INS-1 β-cell line, we hypothesized that activation of the redox sensitive protein TXNIP is involved in ceramide-induced β-cell dysfunction. Incubation of INS-1 cells and primary islets with C2-ceramide (*N*-acetyl-sphingosine) downregulated insulin and PDX-1 expression and increased β-cell apoptosis. Ceramide treatment induced a time dependent increase in TXNIP gene expression accompanied by activation of nuclear factor (NF)-κB and reduced mitochondrial thioredoxin (TRX) activity. Pretreatment with sulfo-N-succinimidyl oleate (SSO), an irreversible inhibitor of the scavenger receptor CD36, blocked ceramide-induced up-regulation of TXNIP expression and activity of NF-κB. Blockade of NF-κB nuclear translocation by the peptide SN50 prevented ceramide-mediated TXNIP induction. Furthermore, SSO also attenuated ceramide-induced early loss of insulin signaling and apoptosis. Collectively, our results unveil a novel role of CD36 in early molecular events leading to NF-κB activation and TXNIP expression. These data suggest that CD36 dependent NF-κB-TXNIP signaling contributes to the ceramide-induced pathogenesis of pancreatic β-cell dysfunction and failure.

1. Introduction

Type 2 diabetes (T2D) is a heterogeneous syndrome resulting from a progressive impairment of beta-cell insulin secretion and insulin resistance in target tissues. In diabetic conditions, beta-cells are unable to sustain a compensatory response, leading to beta-cell dysfunction and death [1]. Impaired insulin secretion coupled to T2D is believed to be caused by several abnormalities which include mitochondrial dysfunction, oxidative stress, ER stress, hyperglycemia (glucotoxicity), dyslipidemia (lipotoxicity), and the combination of both (glucolipotoxicity) [2–8]. Studies with the Zucker diabetic fatty (ZDF) rat, an obesity-induced diabetic animal model, indicate that elevated levels of FFA cause ceramide accumulation which destroys beta-cells by apoptosis [9]. Cellular ceramide content can also be increased via expression of inflammatory cytokines [10,11]. Plasma ceramides are also increased in genetically obese diabetic mice and obese subjects with T2D [12,13]. These observations suggest that ceramide accumulation plays a major role in the development of diabetes. Furthermore, inflammatory cytokine production caused by ceramide-induced activation of the inflammasome leads to pulmonary edema [14].

Thioredoxin-interacting protein (TXNIP) affects the expression of genes by altering the cellular redox state that links to ER stress, inflammasome and beta-cell death [15–17]. TXNIP deficiency protects against the mitochondrial death pathway but against ER-stress-mediated lipoapoptosis [18]. Whether ceramide promotes TXNIP expression in pancreatic beta-cell has not been determined. In order to explore the effect of ceramide, it is tempting to speculate that fatty acid transporter/cluster determinant 36 (FAT/CD36), a class B scavenger receptor, may be involved in the induction of beta-cell dysfunction by ceramide. CD36 binds a variety of ligands including FFA, functions in many different biological processes, and is localized in the secretory granules of beta-cells [19,20]. Forced expression of CD36 in beta-cells increases the uptake of fatty acids and leads to metabolic and functional alterations [21,22]. Furthermore, CD36 initiates inflammatory signaling in adipocytes and macrophages in diet-induced obesity and hyperlipidemias [23].Therefore, it was investigated whether CD36 mediates TXNIP activation and the subsequent induction of beta-cell dysfunction by ceramide.

* Corresponding author.
** Correspondence to: K.C. Won, Department of Internal Medicine, Yeungnam University College of Medicine, 170, Hyunchung-ro, Namgu, Daegu 705-703, South Korea.
E-mail address: kcwon@med.yu.ac.kr (K.C. Won).

http://dx.doi.org/10.1016/j.bbadis.2015.08.009
0925-4439/

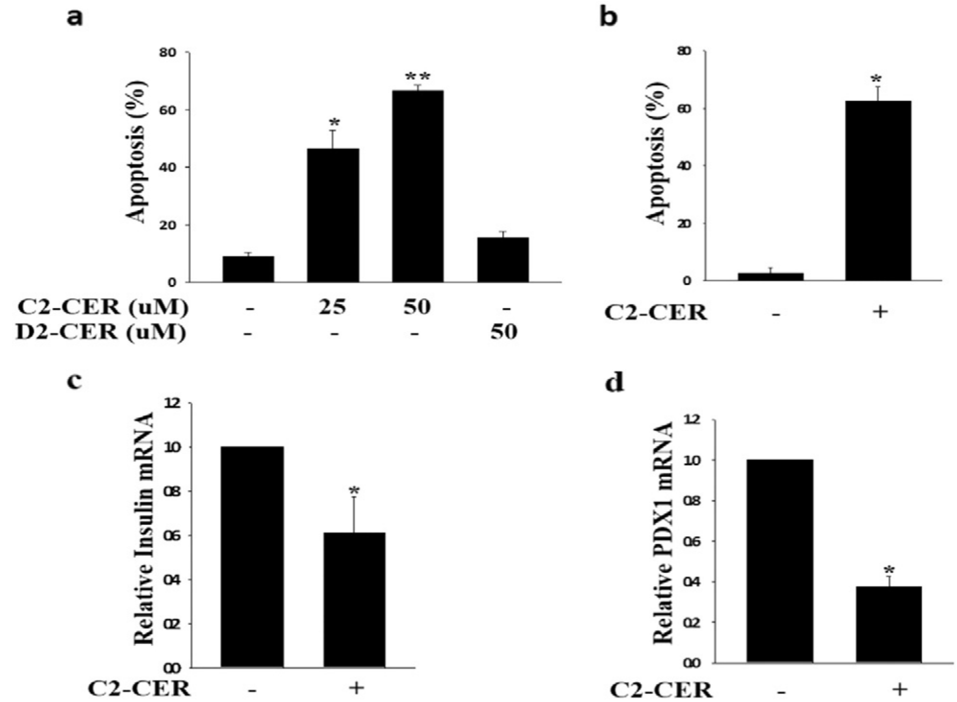

Fig. 1. Effects of ceramide on beta-cell apoptosis, insulin and PDX1 gene expression. (a) INS-1 cells were treated with indicated concentrations of C2-ceramide and inactive analog of C2-ceramide (C2-dihydroceramide) for 24 h, and apoptosis was assessed by fluorescence microscopy. Data represent means ± SEM of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.001$ vs control. (b) Rat islet cells were treated with C2-ceramide (50 μM) for 24 h, and death was assessed by fluorescence microscopy. $^{*}P < 0.005$ vs control. (c, d) INS-1 cells were treated with C2-ceramide for 24 h. Insulin and PDX1 mRNA levels were quantified by real-time RT-PCR. Results are presented as means ± SEM of three independent experiments. $^{*}P < 0.05$ vs control (c); $^{*}P < 0.001$ vs control (d).

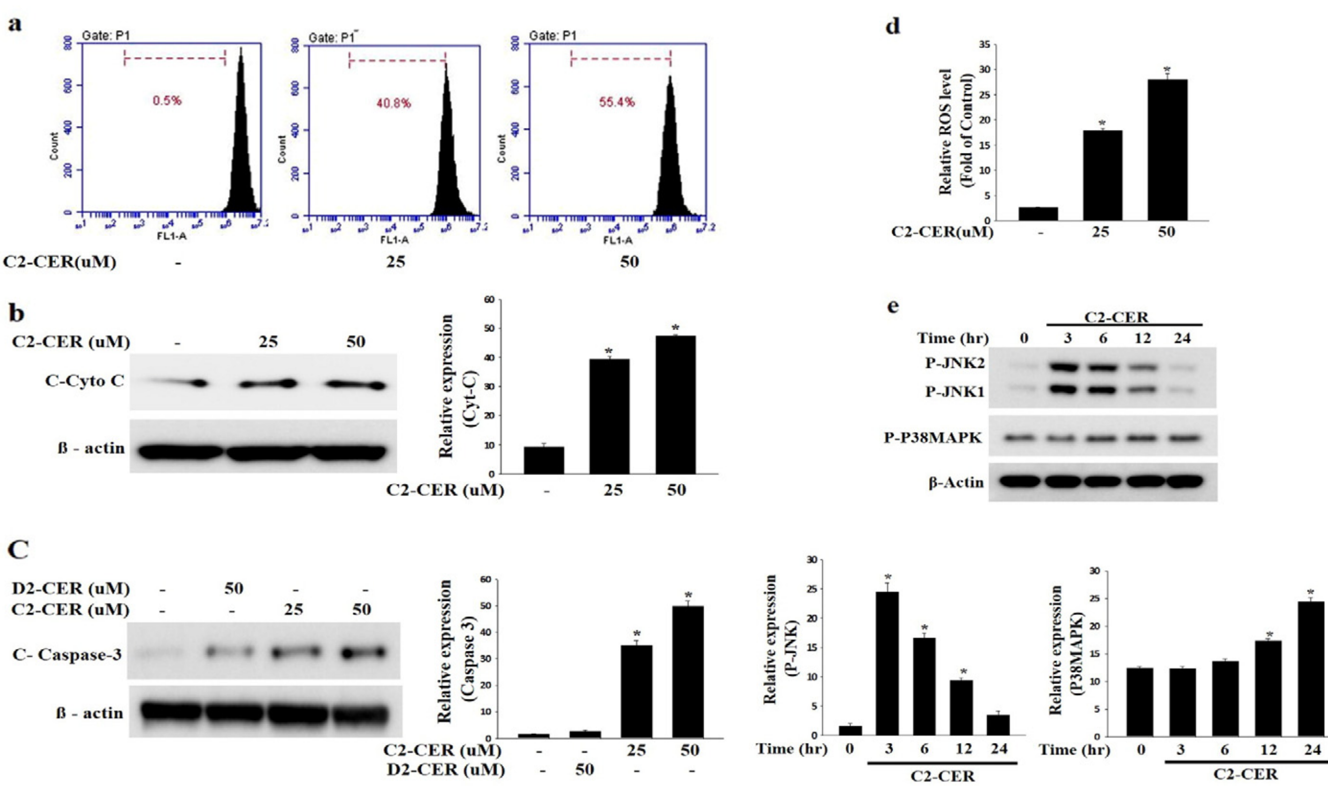

Fig. 2. Ceramide effect on mitochondrial dysfunction. (a) INS-1 cells were treated with C2-ceramide for 3 h and mitochondrial membrane potential was analyzed by FACs using DiO6 dye. (b–c) INS-1 cells were treated with indicated concentrations of C2-ceramide and inactive analog of C2-ceramide (C2-dihydroceramide) for 24 h. Representative protein expression of cytochrome c in cytosol and cleaved caspase 3 were analyzed by Western blot. β-Actin used as a loading control. (d) Cellular ROS production was detected by fluorescence microscopy using 10 μM/L DCF-DA. (e) Cells were treated with C2-ceramide (50 μM) for different time periods and phospho form of JNK, p38MAPK was analyzed by Western blot analysis. Equal loading was confirmed by using β-actin. Results are presented as means ± SEM of three independent experiments. $^{*}P < 0.005$ vs control.

2. Materials and methods

2.1. Cell culture, chemicals

The INS-1 rat insulinoma cell line (INS-1) was cultured in 5% CO_2 at 37 °C in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin. Pancreatic islets were isolated from male Sprague–Dawley rats by collagenase digestion, handpicked and dispersed in an RPMI medium. C2-ceramide and C2-dihydroceramide and DiOC6 were purchased from Sigma-Aldrich (St. Louis, MO, USA), sulfo-N-succinimidyl oleate (SSO) from Cayman Chemical (Ann Arbor, MI, USA).NF-κB inhibitor (sc-3060) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2.2. Apoptosis and mitochondrial functional assay

Apoptosis was determined by TUNEL in-situ cell death detection kit (Roche, Basal, Switzerland). Briefly, cells were serum starved and then treated with various doses of C2-ceramide and C2-dihydroceramide (D2-ceramide) (50 μM) for 24 h. Islets was treated with C2-ceramide (50 μM) for 24 h. Upon completion of treatment, cells were processed further, according to the manufacturer's instructions and image was captured using fluorescence microscopy. Percentage of cell death was quantified by ImageJ software (National Institutes of Health). For the analysis of mitochondrial membrane potential, cells were treated with C2-ceramide (50 μM) for 3 h and then labeled with 10 nM DiOC6 for 5 min at 37 °C. After incubation, the cells were washed once and resuspended in PBS for flow cytometry. Cellular reactive oxygen species were measured using the peroxide sensitive fluorescent probe 2, 7-dichlorodihydrofluorescein diacetate (DCF-DA; Molecular Probes, Carlsbad, CA, USA). Upon completion of treatment, cells were washed with PBS and then incubated in the dark for 15 min with 10 μM/L DCF-DA at 37 °C, and then visualized under a fluorescence microscope. ROS levels were calculated as percentage increases versus control. Mitochondrial dysfunction and apoptosis were also confirmed by Western blot analysis of cytochrome c release and cleaved caspase 3 activation. Mitochondrial localization of TXNIP was visualized using mitochondrion-selective probe CMXRos (200 nM final concentration; MitoTracker Red, Molecular Probes, Eugene, OR).

2.3. Real-time PCR

Total RNA was isolated using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using the First strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Real-time PCR was performed using specific gene primers: 5′ - ACC CAA GTC CCG TCG TGA AGT - 3′ (forward) and 5′ - CCA GTT GGT AGA GGG AGC AGA TG - 3′ (reverse) for insulin; 5′ - GGC TTA ACC TAA ACG CCA CA - 3′ (forward) and 5′ - GGG ACC GTC CAA GTT TGT AA - 3′ (reverse) for pancreatic duodenal homeobox-1 (PDX-1); and 5′ - CGAG TCAAAGCCGTCAGGAT - 3′ (forward) and 5′ - TTCATAGCGCAAGTAGTC CAAGGT - 3′ (reverse) for TXNIP. Transcript values were normalized to a signal obtained with the β-actin and expressed as fold differences versus controls.

2.4. NF-κB-p65 DNA-binding activity

Activation of the NF-κB complex was quantified by using an ELISA-based Cayman's p65 transcription factor assay (Cayman Chemicals, Ann Arbor, MI, USA). Briefly, oligonucleotides that contained a consensus

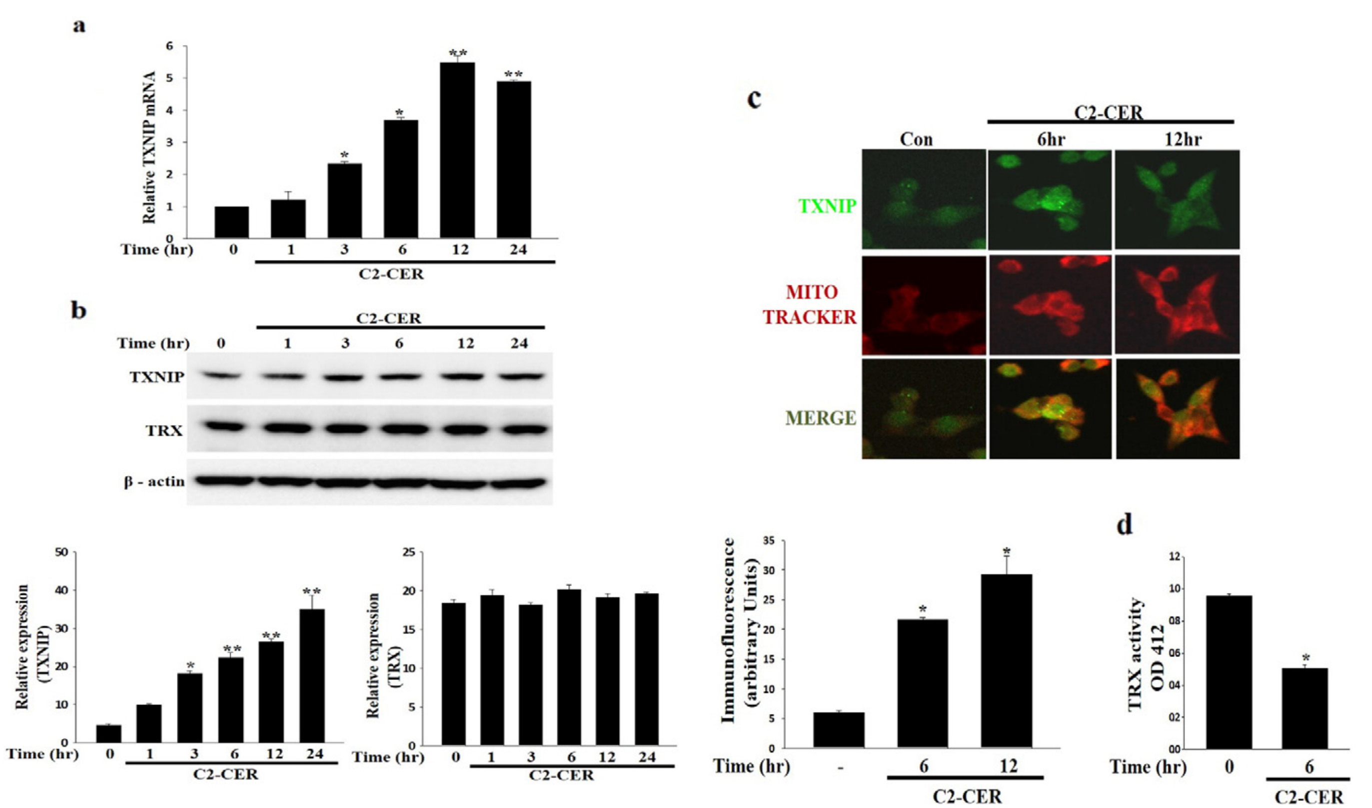

Fig. 3. Ceramide induces TXNIP mRNA, protein expression and reduces thioredoxin activity. (a) TXNIP expression was regulated by C2-ceramide in a time dependent manner measured by RT-PCR. TXNIP mRNA increases as early as 3 h of incubation with C2-ceramide. *$P < 0.005$ and **$P < 0.001$ vs control. Results are expressed as means ± SEM. (b) The protein expression of TXNIP was measured by immunoblotting. Relative protein units were calculated with reference to β-actin. *$P < 0.01$ and **$P < 0.005$ vs control. (c) Mitochondrial localization of TXNIP was visualized using mitochondrion-selective probe CMXRos. (d) Thioredoxin activity was measured by insulin reduction assay after C2-ceramide treatment for 6 h. Data represent means ± SEM of three independent experiments. *$P < 0.005$ vs ceramide.

p65 binding site were immobilized in the wells of plates and incubated with nuclear extracts. The plate was washed extensively according to the manufacturer's instructions and incubated with a specific primary antibody, followed by a secondary antibody conjugated to horseradish peroxidase (HRP) that was used to provide a sensitive colorimetric readout at 450 nm. Nuclear extract was fractionated using the NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Fisher Scientific Inc., Rockford, USA) according to the instructions of the supplier.

2.5. Thioredoxin activity

The mitochondrial fraction was extracted using a mitochondria isolation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The protein concentration was obtained using the Bradford protein assay. Thioredoxin activity in cell lysates was measured by the method of Chen et al. [24].

2.6. Western blotting

Protein lysates were prepared using a lysis buffer (20 mM/L Tris, pH 7.4, 10 mM/L Na_4P_2OH, 100 mM/L NaF, 2 mM/L Na_3VO_4, 5 mM/L EDTA, pH 8.0, 0.1 mM/L PMSF, 1% NP-40) containing proteinase and phosphatase inhibitors. Proteins were resolved by running on a NuPAGE 4–12% Bis–Tris gel (Invitrogen) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated with primary antibodies: phospho JNK, total JNK, phospho p38 MAPK, total p38 MAPK, cleaved caspase 3, phospho AKT, total AKT, phospho p65 (Cell Signaling Technology, Danvers, MA, USA), total p65 (Santa Cruz, Dallas, TX, USA), TXNIP, thioredoxin, and actin (Abcam, Cambridge, UK), followed by washing and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive proteins were detected using ECL reagents (ECL Plus; Amersham, GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Band densities were measured using ImageJ software (NIH).

2.7. Immunofluorescence

Cells were fixed in 2% paraformaldehyde for 15 min and then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. Phospho NF-κB-p65 primary antibody was applied overnight and then was incubated for 1 h with the secondary antibody Alexa-Fluor488 (Invitrogen, Eugene, OR, USA). Cells were observed through an Olympus Fluoview™ FV1000 confocal microscope. Immunofluorescence images were quantified by ImageJ software (NIH).

2.8. Determination of insulin content

Intracellular insulin content was measured by using a rat insulin radioimmunoassay kit (Millipore; EZRMI-13K, Billerica, MA) according to the manufacturer's instructions.

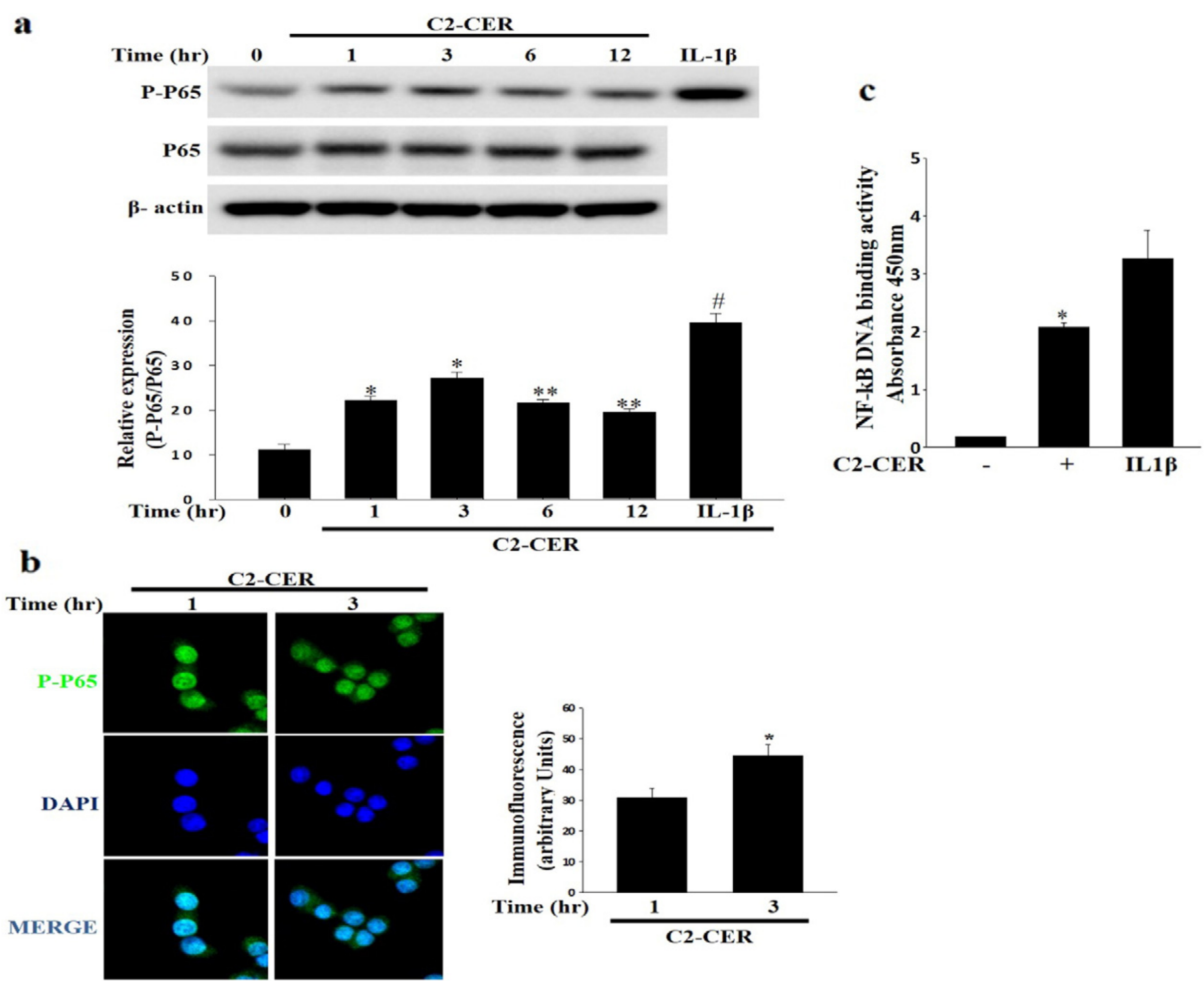

Fig. 4. Ceramide effect on NF-κB activation. (a) INS-1 cells were treated with C2-ceramide (50 μM) for different time periods and expression of phospho-P65 and total form of P65 was analyzed by immunoblotting. IL1β used as a positive control. $^{*}P < 0.01$; $^{**}P < 0.005$; $^{\#}P < 0.005$ vs control (b) The cellular localization of phospho-P65 and nuclei stained with DAPI (blue) were examined using a confocal microscope. $^{*}P < 0.01$ vs 1 h (c) The DNA-binding activity of p65 was measured by enzyme-linked immunosorbent assay after C2-ceramide treatment for 90 min. Results are expressed as means ± SEM. $^{*}P < 0.001$ vs control.

2.9. Statistical analysis

Statistical significance was determined with the Student's *t*-test or by analysis of variance (ANOVA) as appropriate. $P < 0.05$ was defined as statistically significant. All experiments were repeated independently three or more times.

3. Results

3.1. Induction of beta-cell apoptosis by ceramide

C2-ceramide induced a concentration-dependent apoptotic response in beta-cells (Fig. 1a). D2-ceramide, an inactive ceramide analog, was not effective. C2-ceramide (50 μM) also induced a cytotoxic effect in isolated rat islets (Fig. 1b). Ceramide-induced downregulation of beta-cell survival was associated with suppression of insulin mRNA and PDX1 mRNA expression (Fig. 1c–d). Ceramide also induced a significant loss of total cellular insulin content compared with controls (Supplement Fig. 1).

3.2. Induction of mitochondrial dysfunction by ceramide

Treatment of INS-1 cells with C2-ceramide caused a collapse in the mitochondrial membrane potential (Δψ) followed by a release of cytochrome c into the cytosol, reflecting the activation of the permeability transition pore (PTP), a key event in mitochondrial-dependent apoptotic cell death (Fig. 2a–b). Release of cytochrome c into the cytosol leads to caspase-3 activation (Fig. 2c). Furthermore, the total intracellular ROS level, a crucial factor promoting beta-cell apoptosis, was increased by C2-ceramide (Fig. 2d). Consistent with this finding, C2-ceramide treatment increased the activation of stress kinases JNK and P38 MAPK (Fig. 2e) but not ERK (data not shown). These findings suggest that ceramide induces apoptosis by inducing mitochondrial dysfunction.

3.3. Induction of TXNIP expression and inhibition of thioredoxin by ceramide

Treatment of INS-1 cells with C2 ceramide increased the expression of TXNIP (Fig. 3a) without any change in the expression levels of thioredoxin (Fig. 3b). However, thioredoxin plays a vital role in redox imbalance induced by mitochondrial stress by repairing the reduction of proteins by cysteine thiol–disulfide exchange. To clarify whether ceramide-induced TXNIP regulates thioredoxin activity, mitochondrial shuttling of TXNIP was assessed with MitoTracker Red, a mitochondria-specific probe, by immunofluorescence (Fig. 3c). Ceramide treatment induced mitochondrial localization of TXNIP with inhibition of thioredoxin activity (Fig. 3d). Therefore, ceramide-induced activation of TXNIP induced a negative redox balance by negatively regulating thioredoxin activity.

3.4. Activation of NF-κB by ceramide

We speculated that ceramide-induced up-regulation of TXNIP expression was associated with increased NF-κB activation, an important nuclear transcription factor in the regulation of both the inflammatory response and cell survival. Ceramide-treated INS-1 cells showed an early increased level of phospho-NF-κB-p65 with nuclear translocation of the p-p65 subunit confirmed by Western blot and immunofluorescence analysis (Fig. 4a–b). Additionally, elevated DNA binding and transcriptional activities of p-NF-κB-p65 were observed by ELISA-based transcription factor assay (Fig. 4c).

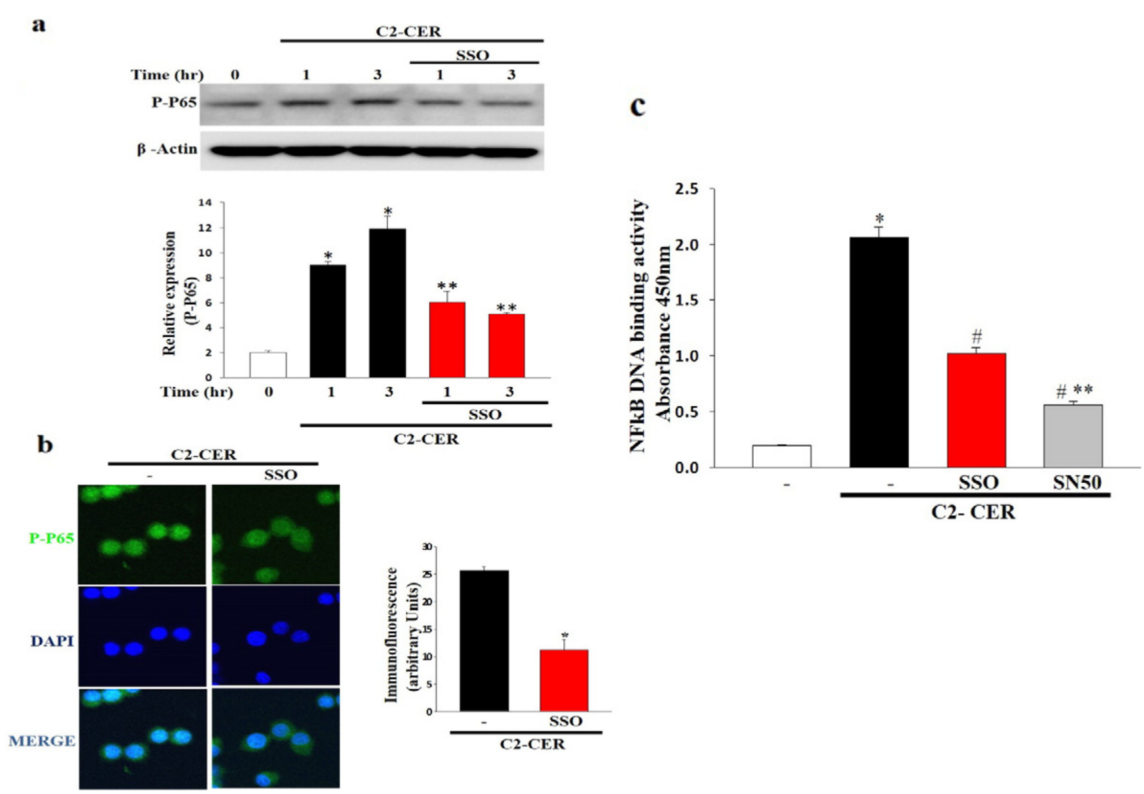

Fig. 5. Effect of CD36 inhibition on ceramide induced NF-κB activation. (a) CD36 inhibitor SSO inhibits ceramide-induced p-P65 activation. Cells were pretreated with SSO (200 μM) for 1 h and then exposed to C2-ceramide for indicated time periods. Expression of phospho-P65 was analyzed by immunoblotting.*$P < 0.005$ vs control; **$P < 0.05$ vs ceramide (b) SSO (200 μM) pretreatment inhibits phospho-p65 nuclear localization examined by a confocal microscope after 1 h of C2-ceramide treatment. (c) Effect of SSO (200 μM) and NF-κB inhibitor SN50 on C2-ceramide induced p-P65 DNA-binding activity. Briefly, cells were pretreated with SSO (200 μM) and NF-κB inhibitor peptide SN50 (36 μM) for 1 h and then exposed to C2-ceramide for 90 min. DNA-binding activity of NF-κB p-P65 was measured by ELISA. Values represent means ± SEM and statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. *$P < 0.001$ vs control; #$P < 0.001$ vs ceramide and **$P < 0.001$ vs SSO.

Early activation and transcription-dependent regulation of NF-κB by ceramide imply that ceramide-induced NF-κB acts upstream of TXNIP expression, in contrast to the report that TXNIP induces NF-κB in retinal capillary endothelial cells under diabetic conditions [25]. In keeping with this view, ceramide-induced NF-κB activation evoked a significant pro-apoptotic role in beta-cells.

3.5. Inhibition of CD36 prevents ceramide-induced NF-κB activation and reduces TXNIP expression

The irreversible CD36 inhibitor sulfo-N-succinimidyl oleate (SSO) inhibited ceramide-induced activation and nuclear translocation of NF-κB (Fig. 5a–b). We further characterized SSO effect on NF-κB DNA binding activity. As shown in Fig. 5c, SSO treatment significantly decreased NF-κB DNA binding activity in ceramide-treated cells. In addition, a cell permeable NF-κB inhibitor peptide SN5O blocked NF-κB activity by ceramide.

We hypothesized that if SSO blocked ceramide stimulated NF-κB, then the expression of TXNIP should be inhibited. As predicted, SSO (200 μM) pretreatment rapidly decreased the TXNIP mRNA expression levels compared with ceramide-treated cells at different time periods (Fig. 6a). In addition, TXNIP protein translation was also decreased by SSO treatment (Fig. 6b). Furthermore, we examined the possibility that TXNIP-mediated decrease in thioredoxin activity would be prevented by SSO treatment. Similarly, thioredoxin activity was markedly recovered from SSO-treated cells as compared with ceramide-treated cells (Fig. 6c). To a similar extent, pretreatment of cells with SN50 significantly reduced ceramide-induced TXNIP gene expression (Fig. 6d).Moreover, TXNIP protein expression was also reduced by SN50 treatment (Fig. 6e). Taken together, these results demonstrate that ceramide-mediated TXNIP expression was regulated by CD36–NF-κB pathways.

3.6. Inhibition of CD36 prevents ceramide-induced TXNIP expression on islet cells

Pretreatment of isolated islets with SSO inhibited ceramide-induced TXNIP expression in a time dependent manner (Fig. 7a) to a similar extent to that observed in INS-1 cells. SSO treatment significantly decreased TXNIP protein expression (Fig. 7b). Taking together, our results suggest that CD36 signaling contribute to ceramide-induced beta cell dysfunction.

3.7. Inhibition of CD36 prevents the reduction of insulin, PDX1 mRNA and apoptosis by ceramide

In an attempt to establish the effects of ceramide on insulin signaling events, Akt activity was measured. An inverse correlation between ceramide and Akt activity has been documented in several studies [26–29]. To this end, INS-1 cells were treated with SSO and then exposed to ceramide. SSO pretreatment markedly restored the Akt activity (Fig. 8a). SSO also significantly reversed the inhibitory effect of ceramide

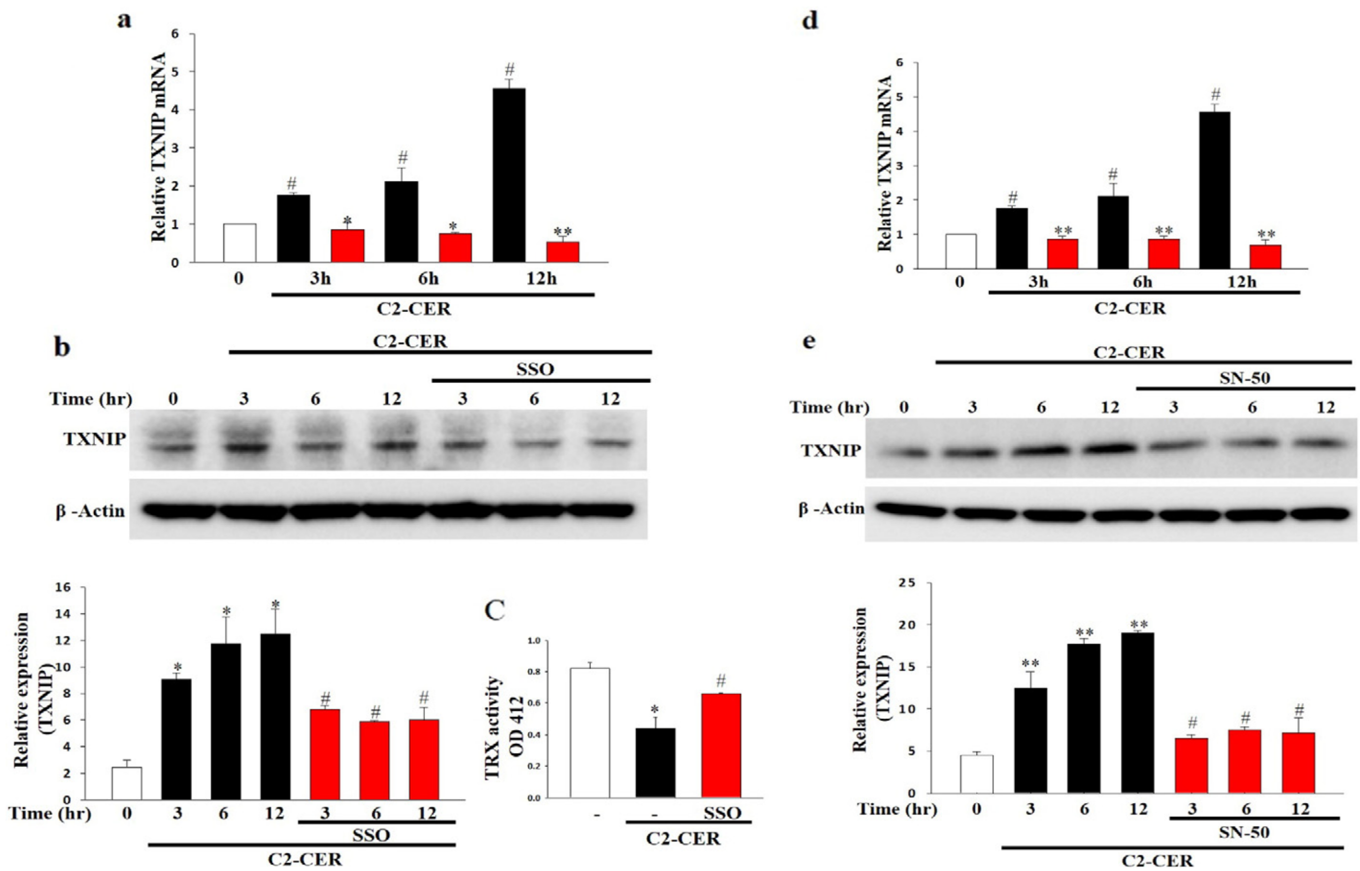

Fig. 6. Inhibitory effect of SSO on ceramide induced TXNIP mRNA, protein and thioredoxin activities. (a) Inhibition of CD36 blocks ceramide-induced expression of TXNIP mRNA. Data represent the mean ± SEM of three independent measurements. $^{\#}P < 0.05$ vs control; $^{*}P < 0.05$;$^{**}P < 0.005$ compared with ceramide under the same treatment condition as determined by two-way ANOVA followed by Tukey's post hoc test. (b) Inhibition of CD36 reduced ceramide-induced TXNIP protein expression. Values represent means ± SEM of three independent experiments. $^{*}P < 0.05$ vs control, $^{\#}P < 0.05$ vs ceramide (c) SSO pretreatment prevents ceramide-induced reduction of thioredoxin activity. Cells were pretreated with SSO (200 μM) for 1 h and then exposed to C2-ceramide for 6 h. Results are expressed as means ± SEM. $^{*}P < 0.05$ vs control; $^{\#}P < 0.05$ vs relative ceramide-treated group. (d) Inhibition of NF-κB by SN50 blocks ceramide-induced TXNIP mRNA. Results are presented as means ± SEM of three independent experiments. $^{\#}P < 0.05$ vs control; $^{**}P < 0.005$ compared with ceramide under the same treatment condition and (e) SN50 blocks ceramide induced TXNIP protein expression. Values represent means ± SEM of three independent experiments. Statistical significance was determined by two-way ANOVA followed by Tukey's post hoc test. $^{**}P < 0.05$ vs control, $^{\#}P < 0.05$ vs relative ceramide-treated group.

on insulin and PDX1 mRNA expression (Fig. 8b–c). Finally, we wanted to correlate the effects of SSO on beta cell survival. Interestingly, SSO treated cells had a much smaller apoptotic response to ceramide, concomitant with a marked inhibition of cleaved caspase 3 expression (Fig. 8d–e). Together, these results suggest that CD36 plays a key role in ceramide-induced beta-cell apoptosis.

4. Discussion

In this study, we demonstrated for the first time that ceramide induces the expression of TXNIP, an endogenous inhibitor of thioredoxin in pancreatic beta cells. CD36 initiated activation of NF-κB acts as a critical factor in ceramide-induced TXNIP expression. Furthermore, blockade of CD36 increases insulin signaling and prevents the development of β-cell failure.

Overwhelming evidence suggests that abnormal ceramide signaling plays a critical role in the pathophysiology of beta-cell failure during T2D. In agreement with what has been previously found, we observed an apoptotic response to ceramide in the pancreatic beta cell line as well as isolated islet cells. The inactive ceramide analog (D2-ceramide) failed to induce an apoptotic effect. The results of multiple studies with diverse cell lines, in rat and mouse islet preparations, and more importantly in human islet cells, also link endogenous production and accumulation of ceramide to increased rates of apoptosis [30–32]. Additionally, we found reduced insulin gene expression by ceramide accompanied with the reduction of PDX1 mRNA expression, a critical regulator of insulin gene expression as well as beta-cell development, differentiation and survival. In line with this, Kelpe et al. [33] found the generation of ceramide through the action of palmitate-reduced insulin gene expression. With this functional characterization, we also tested the effect of ceramide on intracellular insulin content in INS-1 as well as in isolated beta-cells. Of note, the intracellular insulin content was significantly decreased in insulin producing cells, suggesting pronounced cytotoxic effects of ceramide (Supplement 1). Most strikingly, it is closely integrated with mitochondria, which play an important role in apoptotic cell death by multiple stimuli. Ceramide substantially reduced the mitochondrial membrane potential followed by mitochondrial release of cytochrome c, reflecting activation of the mitochondrial permeability transition fore (PTP), leading to the death of the beta-cell. Under the same conditions, ceramide induced the accumulation of intracellular reactive oxygen species (ROS) which are known to be cytotoxic and to rapidly activate stress kinases.

In response to oxidative stress, TXNIP translocates to mitochondria where it directly binds to and inhibits the anti-oxidative protein thioredoxin, causing an increase in beta-cell apoptosis through the intrinsic mitochondrial death pathway [34]. In contrast, our data show that ceramide-induced TXNIP mRNA expression associates with the increased TXNIP protein expression in the mitochondria with reduced thioredoxin activity. Given that TXNIP expression is related to a diverse number of pathological functions, the identification of molecules that can modulate TXNIP expression in response to ceramide requires further study. Identification of such molecules may lead to the development of a new drug target. In this context, it is interesting to note that ceramide-induced activation of NF-κB is accompanied by increased TXNIP expression.

Ceramide causes NF-κB activation [35,36]. Studies also suggest that TXNIP contains binding sites for NF-κB [37]. Induction of TXNIP expression by glucocorticoid is mediated by p38/MAPK in beta-cell death [38]. It is likely that P38/MAPK levels rise in response to ceramide and inhibition of p38/MAPK does not inhibit TXNIP induction (Supplement 3). Therefore, activation of NF-κB is thought to be a critical factor contributing to the induction of TXNIP expression by C2-ceramide in beta-cells.

Recent studies in monocytes showed that palmitate-induced expression of CD36 enhances ceramide accumulation [39]. We found that treatment with C2-ceramide increased CD36 protein levels (Supplement 2). In addition, CD36 is increased in rodent models of insulin resistance and in skeletal muscle of obese humans [40,41]. Moreover CD36-deficient macrophages have reduced NF-κB activation and reduced release of interleukin (IL)-1b and tumor necrosis factor (TNF)-α [42]. We show that SSO inhibits ceramide-induced TXNIP expression. Interestingly, inhibition of CD36 by SSO significantly reduced ceramide-induced NF-κB activation which is upstream of TXNIP induction. Along these lines, inhibition of NFκB active complex nuclear translocation by the cell-permeable peptide SN-50 significantly prevented ceramide-induced TXNIP expression. Consistent with our findings, previous reports provide evidence correlating reduced levels of activated NF-κB in CD36-knock-out mice and monocyte-derived macrophages from CD36-deficient patients [43,44]. Interestingly, inhibition of CD36 by SSO reduced TXNIP protein expression with the significant restoration of mitochondrial thioredoxin-2 activity. Furthermore, mitochondrial membrane potential loss was partially recovered by SSO (data not shown).

A recent study by Vandanmagsar et al. [45] reported that lipotoxicity-associated increases in intracellular ceramide induce caspase-1 cleavage in macrophages and adipose tissue through NLRP3 inflammasome

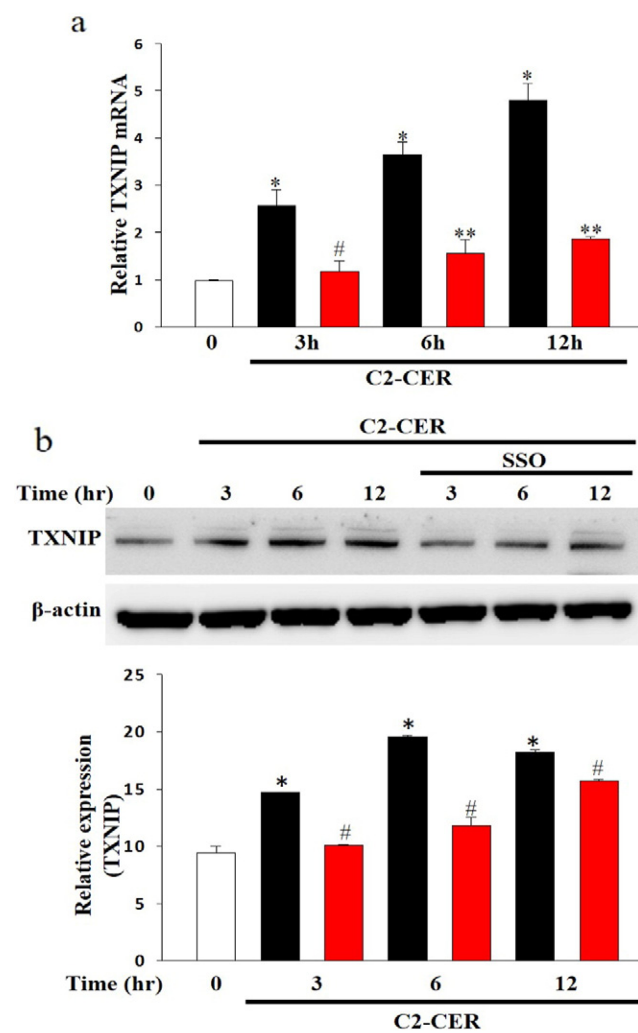

Fig. 7. Inhibition of CD36 blocks ceramide-induced TXNIP mRNA and protein expression in rat islets. (a) Rat islet clusters were isolated and treated with C2-ceramide (50 μM) in the presence or absence of SSO (500 μM) for the indicated time points. TXNIP gene expression was assessed by RT-PCR. Data are expressed as means ± SEM. *$P < 0.05$ vs control; #$P < 0.005$, **$P < 0.001$ vs relative ceramide-treated group as determined by two-way ANOVA followed by Tukey's post hoc test. (b) Isolated islets were pretreated with SSO (500 μM) for 1 h and then exposed to C2-ceramide for the indicated time points. The cell lysates were analyzed by Western blotting with TXNIP or β-actin antibodies. Results are presented as means ± SEM of three independent experiments. *$P < 0.005$ vs control; #$P < 0.001$ vs ceramide-treated group.

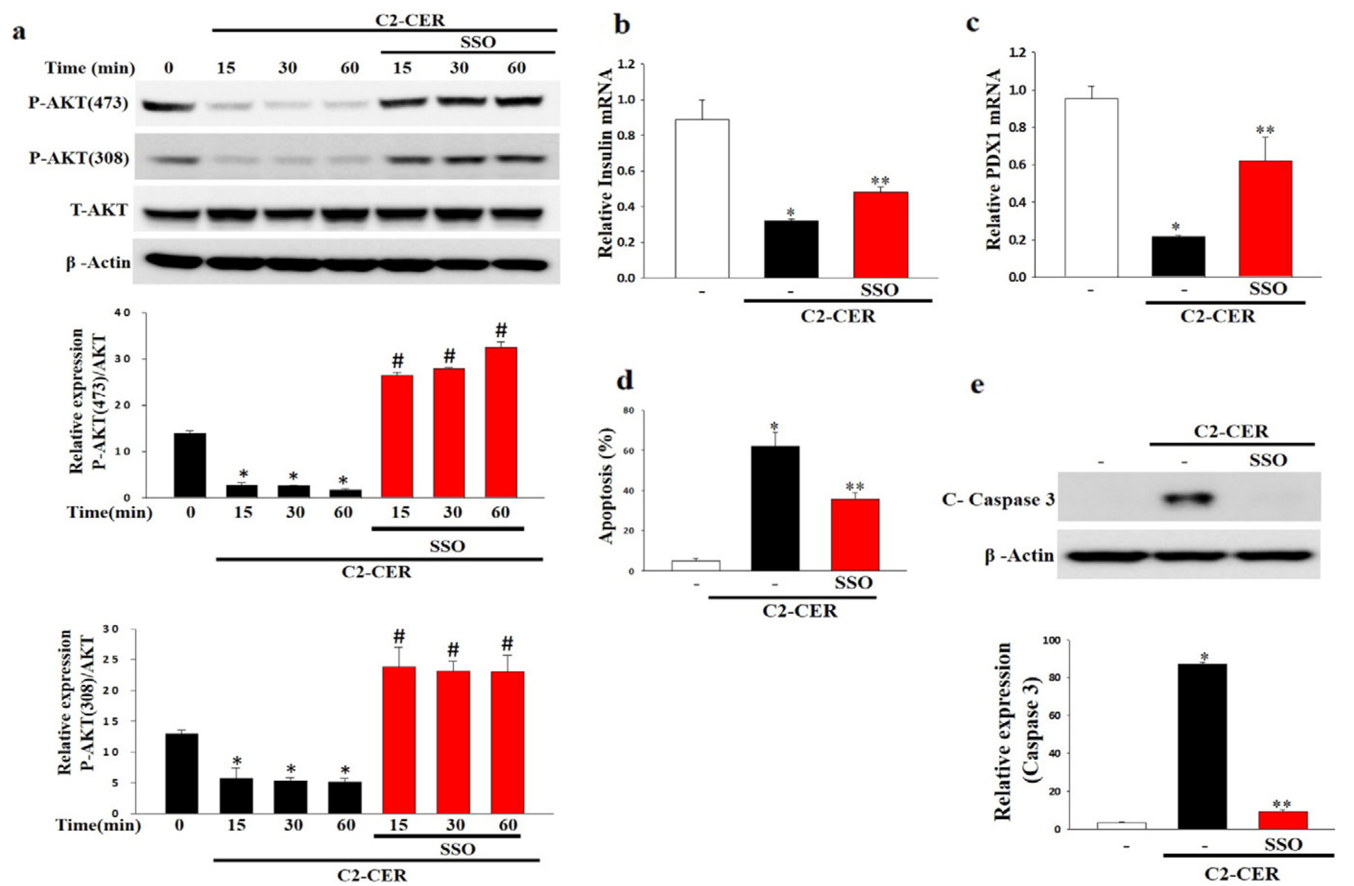

Fig. 8. Representative Western blots showing protein expression of (a) phospho and total form of Akt after C2-ceramide and SSO treatment. SSO was added 1 h prior to the incubations with C2-ceramide. *$P < 0.005$ vs control; $^{\#}P < 0.001$ vs ceramide. (b–c) SSO treatment attenuated ceramide reduced insulin and PDX1 gene expression. *$P < 0.001$ vs control; **$P < 0.05$ vs ceramide. (d) Inhibition of CD36 by SSO attenuates ceramide-induced apoptosis. INS-1 cells were treated with SSO for 1 h and then exposed to C2-ceramide for another 24 h. Cell death was assessed by fluorescence microscopy. Values represent means ± SEM. *$P < 0.01$ vs control; **$P < 0.05$ vs ceramide. (e) Cleaved caspase 3 was analyzed by Western blot. *$P < 0.005$ vs control; **$P < 0.001$ vs ceramide-treated group.

activation. In pancreatic beta cells, TXNIP indeed has a role in producing IL-1β through NLRP3 inflammasome activation under ER stress [16]. Moreover, a recent study by Shetty et al. [46] showed that an early CD36-mediated pathogenic inflammasome activation links cholesterol accumulation to the chronic inflammatory process of atherosclerosis. So, future studies are warranted that examine the relationship between CD36 and NLRP3 inflammasome activation in the pancreatic beta cell dysfunction and failure. In addition, proper beta-cell function is dependent on insulin signaling. Akt/PKB expression is reduced by ceramide [47–50]. Taking this into account, we found that ceramide inhibited Akt activation in a time dependent manner while SSO blocked this inhibition. This suggests that Akt activity is important for survival of pancreatic beta-cells. Activation of Akt/PKB directly regulates members of the Bcl-2 family and decreases the percentage of apoptotic cells by ceramide [51]. In addition, activation of Akt/PKB inhibits early apoptotic events by maintaining mitochondrial integrity [52]. In view of these, activation of mitochondrial signaling may enhance the release of pro-apoptotic factors which may contribute to the induction of apoptosis and downregulation of insulin and PDX1 expression by ceramide. Reduction of CD36 signaling by SSO dampens the oxidative stress dependent signals and increases cell survival.

In conclusion, we highlight the importance of CD36-mediated intracellular signaling in the development of ceramide-induced TXNIP expression and β-cell failure. Our data demonstrate that the inhibition of CD36 reduced ceramide-induced mitochondrial stress, which acts as a major driving force in the progression of T2D. Furthermore, blockade of CD36 increases insulin signaling and prevents the development of β-cell failure. Collectively, this study provides direct evidence that activation of the CD36 signaling in ceramide-induced TXNIP expression is a critical trigger in causing pancreatic damage. Future studies are warranted to determine if CD36 activation is involved in beta-cell inflammasome activation toward type 2 diabetes.

Funding

This research was supported by a grant of the Yeungnam University Medical Center (2014).

Contribution statement

UK contributed to the conception and design of the study, the acquisition, analysis and interpretation of the data and drafting of the article. HWL, JSM and KCW contributed to the conception and design of the study, the analysis and interpretation of the data and the revision of the article.

Transparency document

The Transparency document associated with this article can be found, in the version.

Acknowledgments

The authors thank Dr. Robert A. Harris (Indiana University School of Medicine) for his help with the editing of our manuscript; Dr. In Kyu Lee

(Kyungpook National University School of Medicine) for providing lab space and supplies; Ms. Ye-Jin Seo (Yeungnam University College of Medicine, Daegu, Korea) for technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.08.009.

References

[1] J.L. Leahy, Pathogenesis of type 2 diabetes mellitus, Arch. Med. Res. 36 (2005) 197–209.
[2] R.P. Robertson, J. Harmon, P.O. Tran, V. Poitout, Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes, Diabetes 53 (Suppl. 1) (2004) S119–S124.
[3] G.C. Weir, S. Bonner-Weir, Five stages of evolving beta-cell dysfunction during progression to diabetes, Diabetes 53 (Suppl. 3) (2004) S16–S21.
[4] M. Prentki, C.J. Nolan, Islet beta cell failure in type 2 diabetes, J. Clin. Invest. 116 (2006) 1802–1812.
[5] M. Cnop, N. Welsh, J.C. Jonas, A. Jorns, S. Lenzen, D.L. Eizirik, Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities, Diabetes 54 (Suppl. 2) (2005) S97–S107.
[6] U. Karunakaran, K.G. Park, A systematic review of oxidative stress and safety of antioxidants in diabetes: focus on islets and their defense, Diabetes Metab. J. 37 (2013) 106–112.
[7] J.W. Kim, K.H. Yoon, Glucolipotoxicity in pancreatic beta-cells, Diabetes Metab. J. 35 (2011) 444–450.
[8] U. Karunakaran, H.J. Kim, J.Y. Kim, I.K. Lee, Guards and culprits in the endoplasmic reticulum: glucolipotoxicity and β-cell failure in type II diabetes, Exp. Diabetes Res. 2012 (9) (2012) 639762.
[9] M. Shimabukuro, Y.T. Zhou, M. Levi, R.H. Unger, Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 2498–2502.
[10] N. Welsh, Interleukin-1 beta-induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH2-terminal kinase and the transcription factor ATF2 in the insulin-producing cell line RINm5F, J. Biol. Chem. 271 (1996) 8307–8312.
[11] C.D. Major, Z.Y. Gao, B.A. Wolf, Activation of the sphingomyelinase/ceramide signal transduction pathway in insulin-secreting beta-cells: role in cytokine-induced beta-cell death, Diabetes 48 (1999) 1372–1380.
[12] F. Samad, K.D. Hester, G. Yang, Y.A. Hannun, J. Bielawski, Altered adipose and plasma sphingolipid metabolism in obesity: a potential mechanism for cardiovascular and metabolic risk, Diabetes 55 (2006) 2579–2587.
[13] J.M. Haus, S.R. Kashyap, T. Kasumov, et al., Plasma ceramides are elevated in obese subjects with type 2 diabetes and correlate with the severity of insulin resistance, Diabetes 58 (2009) 337–343.
[14] N. Kolliputi, L. Galam, P.T. Parthasarathy, S.M. Tipparaju, R.F. Lockey, NALP-3 inflammasome silencing attenuates ceramide-induced transepithelial permeability, J. Cell. Physiol. 227 (2012) 3310–3316.
[15] A.H. Minn, C.A. Pise-Masison, M. Radonovich, et al., Gene expression profiling in INS-1 cells overexpressing thioredoxin-interacting protein, Biochem. Biophys. Res. Commun. 336 (2005) 770–778.
[16] C.M. Oslowski, T. Hara, B. O'Sullivan-Murphy, et al., Thioredoxin-interacting protein mediates ER stress-induced beta cell death through initiation of the inflammasome, Cell Metab. 16 (2012) 265–273.
[17] R. Zhou, A. Tardivel, B. Thorens, I. Choi, J. Tschopp, Thioredoxin-interacting protein links oxidative stress to inflammasome activation, Nat. Immunol. 11 (2010) 136–140.
[18] J. Chen, G. Fontes, G. Saxena, V. Poitout, A. Shalev, Lack of TXNIP protects against mitochondria-mediated apoptosis but not against fatty acid-induced ER stress-mediated beta-cell death, Diabetes 59 (2010) 440–447.
[19] R.L. Silverstein, M. Febbraio, CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior, Sci. Signal. 2 (2009) re3.
[20] H. Noushmehr, E. D'Amico, L. Farilla, et al., Fatty acid translocase (FAT/CD36) is localized on insulin-containing granules in human pancreatic beta-cells and mediates fatty acid effects on insulin secretion, Diabetes 54 (2005) 472–481.
[21] T. Wallin, Z. Ma, H. Ogata, et al., Facilitation of fatty acid uptake by CD36 in insulin-producing cells reduces fatty-acid-induced insulin secretion and glucose regulation of fatty acid oxidation, Biochim. Biophys. Acta 1801 (2010) 191–197.
[22] Y.W. Kim, J.S. Moon, Y.J. Seo, S.Y. Park, J.Y. Kim, J.S. Yoon, I.K. Lee, H.W. Lee, K.C. Won, Inhibition of fatty acid translocase cluster determinant 36 (CD36), stimulated by hyperglycemia, prevents glucotoxicity in INS-1 cells, Biochem. Biophys. Res. Commun. 420 (2012) 462–466.
[23] D.J. Kennedy, S. Kuchibhotla, K.M. Westfall, R.L. Silverstein, R.E. Morton, M. Febbraio, A CD36-dependent pathway enhances macrophage and adipose tissue inflammation and impairs insulin signaling, Cardiovasc. Res. 89 (2011) 604–613.
[24] C.L. Chen, C.F. Lin, W.T. Chang, W.C. Huang, C.F. Teng, Y.S. Lin, Ceramide induces p38 MAPK and JNK activation through a mechanism involving a thioredoxin-interacting protein-mediated pathway, Blood 111 (2008) 4365–4374.
[25] L. Perrone, T.S. Devi, K. Hosoya, T. Terasaki, L.P. Singh, Thioredoxin interacting protein (TXNIP) induces inflammation through chromatin modification in retinal capillary endothelial cells under diabetic conditions, J. Cell. Physiol. 221 (2009) 262–272.
[26] E. Hajduch, A. Balendran, I.H. Batty, et al., Ceramide impairs the insulin-dependent membrane recruitment of protein kinase B leading to a loss in downstream signalling in L6 skeletal muscle cells, Diabetologia 44 (2001) 173–183.
[27] S. Stratford, K.L. Hoehn, F. Liu, S.A. Summers, Regulation of insulin action by ceramide: dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein kinase B, J. Biol. Chem. 279 (2004) 36608–36615.
[28] T. Teruel, R. Hernandez, M. Lorenzo, Ceramide mediates insulin resistance by tumor necrosis factor-alpha in brown adipocytes by maintaining Akt in an inactive dephosphorylated state, Diabetes 50 (2001) 2563–2571.
[29] S.A. Summers, L.A. Garza, H. Zhou, M.J. Birnbaum, Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide, Mol. Cell. Biol. 18 (1998) 5457–5464.
[30] X. Lei, S. Zhang, A. Bohrer, S. Bao, H. Song, S. Ramanadham, The group VIA calcium-independent phospholipase A2 participates in ER stress-induced INS-1 insulinoma cell apoptosis by promoting ceramide generation via hydrolysis of sphingomyelins by neutral sphingomyelinase, Biochemistry 35 (2007) 10170–10185.
[31] X. Lei, S. Zhang, A. Bohrer, S. Ramanadham, Calcium-independent phospholipase A2 (iPLA2 beta)-mediated ceramide generation plays a key role in the cross-talk between the endoplasmic reticulum (ER) and mitochondria during ER stress-induced insulin-secreting cell apoptosis, J. Biol. Chem. 50 (2008) 34819–34832.
[32] X. Lei, S. Zhang, B. Emani, S.E. Barbour, S. Ramanadham, A link between endoplasmic reticulum stress-induced β-cell apoptosis and the group VIA Ca^{2+}-independent phospholipase A_2 (iPLA$_2$β), Diabetes Obes. Metab. 12 (2010) 93–98.
[33] C.L. Kelpe, P.C. Moore, S.D. Parazzoli, B. Wicksteed, C.J. Rhodes, V. Poitout, Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis, J. Biol. Chem. 278 (2003) 30015–30021.
[34] G. Saxena, J. Chen, A. Shalev, Intracellular shuttling and mitochondrial function of thioredoxin-interacting protein, J. Biol. Chem. 285 (2010) 3997–4005.
[35] M.P. Boland, L.A. O'Neill, Ceramide activates NFkappaB by inducing the processing of p105, J. Biol. Chem. 273 (1998) 15494–15500.
[36] I. Kitajima, Y. Soejima, I. Takasaki, H. Beppu, T. Tokioka, I. Maruyama, Ceramide-induced nuclear translocation of NF-kappa B is a potential mediator of the apoptotic response to TNF-alpha in murine clonal osteoblasts, Bone 19 (1996) 263–270.
[37] D.L. Ludwig, H. Kotanides, T. Le, D. Chavkin, P. Bohlen, L. Witte, Cloning, genetic characterization, and chromosomal mapping of the mouse VDUP1 gene, Gene 269 (2001) 103–112.
[38] E. Reich, A. Tamary, R.V. Sionov, D. Melloul, Involvement of thioredoxin-interacting protein (TXNIP) in glucocorticoid-mediated beta cell death, Diabetologia 55 (2012) 1048–1057.
[39] D. Gao, C. Pararasa, C.R. Dunston, C.J. Bailey, H.R. Griffiths, Palmitate promotes monocyte atherogenicity via de novo ceramide synthesis, Free Radic. Biol. Med. 53 (2012) 796–806.
[40] D.M. Ouwens, M. Diamant, M. Fodor, et al., Cardiac contractile dysfunction in insulin-resistant rats fed a high-fat diet is associated with elevated CD36-mediated fatty acid uptake and esterification, Diabetologia 50 (2007) 1938–1948.
[41] A. Bonen, N.N. Tandon, J.F. Glatz, J.J. Luiken, G.J. Heigenhauser, The fatty acid transporter FAT/CD36 is upregulated in subcutaneous and visceral adipose tissues in human obesity and type 2 diabetes, Int. J. Obes. 30 (2006) 877–883.
[42] S. Yamashita, K. Hirano, T. Kuwasako, M. Janabi, Y. Toyama, M. Ishigami, et al., Physiological and pathological roles of a multi-ligand receptor CD36 in atherogenesis: insights from CD36-deficient patients, Mol. Cell. Biochem. 299 (2007) 19–22.
[43] A. Kunz, T. Abe, K. Hochrainer, et al., Nuclear factor-kappaB activation and postischemic inflammation are suppressed in CD36-null mice after middle cerebral artery occlusion, J. Neurosci. 28 (2008) 1649–1658.
[44] M. Janabi, S. Yamashita, K. Hirano, et al., Oxidized LDL-induced NF-kappa B activation and subsequent expression of proinflammatory genes are defective in monocyte-derived macrophages from CD36-deficient patients, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1953–1960.
[45] B. Vandanmagsar, Y.H. Youm, A. Ravussin, J.E. Galgani, K. Stadler, R.L. Mynatt, E. Ravussin, J.M. Stephens, V.D. Dixit, The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance, Nat. Med. 17 (2011) 179–188.
[46] F.J. Sheedy, A. Grebe, K.J. Rayner, P. Kalantari, B. Ramkhelawon, S.B. Carpenter, et al., CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation, Nat. Immunol. 14 (2013) 812–820.
[47] S.A. Summers, Ceramides in insulin resistance and lipotoxicity, Prog. Lipid Res. 45 (2006) 42–72.
[48] A. Mora, G. Sabio, A.M. Risco, et al., Lithium blocks the PKB and GSK3 dephosphorylation induced by ceramide through protein phosphatase-2A, Cell. Signal. 14 (2002) 557–562.
[49] R.A. Wolff, R.T. Dobrowsky, A. Bielawska, L.M. Obeid, Y.A. Hannun, Role of ceramide-activated protein phosphatase in ceramide-mediated signal transduction, J. Biol. Chem. 269 (1994) 19605–19609.
[50] G. Arboleda, T.J. Huang, C. Waters, A. Verkhratsky, P. Fernyhough, R.M. Gibson, Insulin-like growth factor-1-dependent maintenance of neuronal metabolism through the phosphatidylinositol 3-kinase–Akt pathway is inhibited by C2-ceramide in CAD cells, Eur. J. Neurosci. 25 (2007) 3030–3038.
[51] P. Navarro, A.M. Valverde, J.L. Rohn, M. Benito, M. Lorenzo, Akt mediates insulin rescue from apoptosis in brown adipocytes: effect of ceramide, Growth Hormon. IGF Res. 10 (2000) 256–266.
[52] K. Gottlob, N. Majewski, S. Kennedy, E. Kandel, R.B. Robey, N. Hay, Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase, Genes Dev. 15 (2001) 1406–1418.